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Original Article

Comparison of High-Performance Liquid Chromatography and Capillary Electrophoresis in the Screening of Haemoglobinopathies

Shumaila Asghar, Asad Mahmood Abbasi, Jawad Zafar, Rafia Mahmood, Saima Zahir, Maria Khan*****

Department of Hematology, Armed Forces Institute of Pathology/National University of Medical Sciences (NUMS) Rawalpindi Pakistan, *Department of Hematology, Combined Military Hospital, Multan/National University of Medical Sciences (NUMS) Pakistan, **Department of Hematology, Combined Military Hospital, Hyderabad/National University of Medical Sciences (NUMS) Pakistan, ***Department of Hematology, Armed Forces Institute of Transfusion/National University of Medical Sciences (NUMS) Rawalpindi Pakistan

ABSTRACT

Objective: To compare the results of High-Performance Liquid Chromatography and Capillary Electrophoresis in the screening of haemoglobinopathies.

Study Design: Cross-sectional study.

Place and Duration of Study: Department of Haematology, Armed Forces Institute of Pathology, Rawalpindi Pakistan, from May 2020 to Nov 2020.

Methodology: Ninety(90) newly diagnosed patients with haemoglobinopathies were included. Patients with a history of recent blood transfusion (<4 weeks) were excluded. Venous blood samples were taken in EDTA tubes, separated into two aliquots and evaluated in parallel on High-Performance Liquid Chromatography and Capillary Electrophoresis analyzers.

Results: Nine different haemoglobinopathies were encountered in the study sample. The most common condition was beta thalassaemia trait found in 50(55.6%) subjects, while Haemoglobin (Hb) (D) homozygous was the least common, detected in only 1(1.1%) subjects. A significant difference was observed between values in cases of Hb(D) Iran trait; Mean Hb(A2) percentage detected by HPLC was 41.96±1.46% compared to 1.5±0.79% detected by CE (*p*-value<0.001). CE detected a mean of 41.4±1.01% of Hb(D)Iran in the patients of the Hb(D) Iran trait, while High-Performance Liquid Chromatography did not detect this Hb variant (p-value<0.001). A significant difference was also found in cases of Hb(E) trait in percentages of Hb(A2) and Hb(E) variants. High-Performance Liquid Chromatography detected a mean of 31.43±2.84% of Hb(A2) compared to 4.5±0.5% detected by CE (*p*-value=0.003).

Conclusion: Capillary electrophoresis is capable of identifying haemoglobin variants Hb(D) Iran and Hb(E) in cases of Hb(D) Iran trait and Hb(E) trait/Hb(E) homozygous, which HPLC does not pick up.

Keywords: Beta-thalassaemia, Capillary electrophoresis, Haemoglobinopathies, High-Performance liquid chromatography.

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INTRODUCTION

Haemoglobinopathies are one of the most common single-gene disorders worldwide, with increasing incidence in areas endemic to malaria.[1] According to World Health Organization (WHO) statistics, hereditary haemoglobin (Hb) disorders affect nearly 5% of the world's population.[2] The magnitude of the impact of these disorders makes them a global epidemic in the vast majority of areas.[3] Haemoglobinopathies can be quantitative in thalassaemia syndromes or qualitative in different haemoglobin variants, e.g., Hb(S), Hb(C). Beta-thalassaemia affects about 1.5% of the population worldwide, with a 5-7% prevalence in Pakistan.[4]

Identifying Hb variants, disease carriers, and clinically silent but extremely symptomatic Hb modifications must all be part of the screening programmes for detecting haemoglobinopathies.[5,6] This makes proper use of screening and testing procedures critical for diagnosis and treatment. Introduced in the last decade, high-performance liquid chromatography (HPLC) is a reliable tool to identify subtypes and improve the accuracy of Hb(A2) and Hb(F) calculations. The survival time of unknown Hb is compared to that of a calibration system that includes Hb variants: Hb(F), Hb(A2), Hb(S), Hb(C) and Hb(D).[7] Automated capillary zone electrophoresis (CE) is a relatively recent method. United States Food and Drug Administration (USFDA) permitted the Sebia Capillary device (Sebia, Norcross, GA) to assess haemoglobinopathies in 2007.[8,9] CE is an effective tool for detecting monoclonal gammopathies and other protein abnormalities in serum. It is an automated

Correspondence: Dr Shumaila Asghar, Department of Hematology, Armed Forces Institute of Pathology, Rawalpindi Pakistan
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process for separating Hb fractions within a capillary tube using electrophoresis at basic pH.[10]

The present study aims to compare HPLC and CE in screening haemoglobinopathies in the local population. To our knowledge, no existing study has compared the two methods in screening haemoglobinopathies in the Pakistani population. The knowledge gained will help better understand the disease pattern in our population and the diagnostic utility of both methods, resulting in improved treatment and care of affected individuals.

METHODOLOGY

The cross-sectional study was conducted at the Department of Haematology, Armed Forces Institute of Pathology, from May to November 2020, after approval from the Institutional Ethics Review Committee [FC-HEM18-9/READ-IRB/20/356]. Sample size was calculated using the WHO calculator, keeping anticipated population prevalence (P) at 34.23%.[11]

Inclusion Criteria: Newly diagnosed patients of either gender and age group, with various haemoglobinopathies, were included.

Exclusion criteria: Patients with a history of recent blood transfusion (less than four weeks) were excluded.

Ninety (90) newly diagnosed patients of various haemoglobinopathies were included using a non-probability consecutive sampling technique. Informed consent was obtained from all study subjects. Approximately 5 ml of blood was collected from every patient into Ethylenediamine tetra-acetic acid (EDTA) enclosing tubes. For haemoglobinopathy identification, specimens were divided into two small aliquots and investigated on HPLC and CE analyzers within a day according to the manufacturer's instructions. HPLC was performed using BIORAD HPLC, a cation exchange column. This method relates the retention time of unknown Hb fractions to that of a calibrating standard containing Hb variants: Hb(F), Hb(A), Hb(S), and Hb(D). E blood specimens collected in EDTA were lysed with the haemolyzing reagent (provided by the manufacturer) for injection into the HPLC column. Because a bar code reader was absent on our instrument, sample placement was used to identify patients. However, all other procedures, including sample aspiration, lysis, and final pattern, were performed with minimal technical work beyond placing the sample tube in the instrument rack. Adsorbed Hb fractions used a gradient formed by two mobile phases of Bis-tris(hydroxymethyl) amino-methane and one mmol of potassium cyanide with different pH and ionic strength values.

CE was performed using the Sebia Capillary system. The manufacturer's guidelines were followed when performing the analysis. Initial processing of the sample required centrifugation of the whole blood at 5,000 rpm for 5 minutes. The o`verlying plasma was removed, and the erythrocyte pellet was vortexed for 5 seconds. Since the instrument could read the bar code, the instrument performed the identification, sampling, electrophoresis, and production of the electro-phoretogram pattern without further technical intervention. Electrophoresis was performed in an alkaline buffer (pH 9.4) provided by the manufacturer, with separation primarily by the pH of the solution and endosmosis. Hb reactions were measured at 415 nm wavelength. Electrophoretograms were recorded with the location of specific Hb fractions in specific zones. The presence of Hb(A) was required for the appearance of the zone demarcations that help guide the interpretation of structural variants. If b(A) was absent, the sample was re-tested and premixed with a 1:1 mixture of normal control. Quantitative results for such a case were reported from the original sample alone, and the 1:1 mixture was used for qualitative identification only.

Data was analyzed using Statistical Package for Social Sciences (SPSS)-24. Quantitative variables were expressed as mean±SD and qualitative variables were expressed as frequency and percentages. Independent sample t-test was applied to explore the inferential statistics. The p-value of ≤0.05 was considered statistically significant.

RESULTS

This study comprised 90 subjects with various haemoglobinopathies. Of these, 57(63.3%) were male, and 33(36.7%) were female. The mean age of the study subjects was 13.33±14.13 years (range: 06 months to 51 years).

Nine different types of haemoglobinopathies were encountered in the study sample. The most common condition was beta thalassaemia, found in 50/90(55.6%) subjects, while Hb(D) homozygous was the least common, detected in only 1/90(1.1%) subjects (Table-I).

The two analysis methods were compared in screening all identified haemoglobinopathies. A significant difference was observed between values of Hb variants Hb(A2) and Hb(D) detected by HPLC and

those detected by CE in cases of the Hb(D) Iran trait. The mean Hb(A2) percentage detected by HPLC was 41.96±1.46% compared to 1.5±0.79% detected by CE (*p*-value<0.001). Consequently, CE detected a mean of 41.4±1.01% of Hb(D) in the diagnosed patients of Hb(D) Iran trait, while HPLC did not detect this Hb variant (*p*-value<0.001). A significant difference between the two analyzing methods was also found for cases of the Hb(E) trait in detecting the percentages of Hb(A2) and Hb(E) variants (Table-II). HPL detected a mean of 31.43±2.84% of Hb(A2) compared to 4.5±0.5% detected by CE (*p*=0.003). While HPLC was unable to detect any Hb(E) variant in the diagnosed subjects, CE detected a mean of 27.73±1.37% of Hb(E) in subjects diagnosed with the Hb(E) trait (*p*-value<0.001).

For all other diagnosed haemoglobinopathies, no significant difference in the detection of Hb variants was observed between HPLC and CE (Table-III).

Table-I: Type and Frequency of Haemoglobinopathies Diagnosed in Study Subjects (n=90)

Haemoglobinopathy (Type)	n (%)
Beta Thalassaemia trait	50 (55.6)
Beta Thalassaemia major	12 (13.3)
Sickle Cell Anaemia (homozygous)	04 (4.4)
Sickle Cell Trait	03 (3.3)
Hb D Punjab Trait	12 (13.3)
Hb D Homozygous	01 (1.1)
Hb D Iran Trait	03 (3.3)
Hb E Trait	03 (3.3)
Sickle/β-Thalassaemia	02 (2.2)

Table-II: Comparison between High Performance Liquid Chromatography (HPLC) and Capillary Electrophoresis (CE) in Detecting Haemoglobin Variants in Subjects Diagnosed with Hb(D) Iran Trait and Hb(E) Trait (n=90)

Haemoglo-binopathy	Hb Variant %	Analyzer		*p*-value
		HPLC	CE	
Hb(D) Iran Trait	Hb(A)	57.15±1.48	56.28±1.06	0.45
	Hb(A2)	41.96±1.46	1.5±0.79	<0.001
	Hb(F)	0.88±0.04	0.83±0.03	0.086
Hb(E) Trait	Hb(A)	67.66±2.93	66.82±1.22	0.67
	Hb(A2)	31.43±2.84	4.5±0.5	0.003
	Hb(F)	0.87±0.07	0.89±0.07	0.87

DISCUSSION

The comparison between two automated, FDA-approved methods demonstrated the usefulness of both methods in the routine evaluation of patients for the presence of haemoglobinopathies. A good association of measurement of Hb (A) and Hb (F) exists between the two methods,[11] which aligns with our findings. Even though the correlation between HbA2 detection was found, slightly higher values were determined by the HPLC method in the study subjects diagnosed with sickle cell trait. Although statistically insignificant, this difference showed by HPLC may be attributed to the comigration of glycated Hb(S) products along with Hb(A2) peaks. The existence of Hb(S), posttranslational and/or breakdown products in the area of HbA2 by the HPLC technique interferes with correct Hb(A2) estimation.[12,13] Keren *et al*.[14] also endorsed that HbA2 levels were higher by HPLC (Mean: 4+1%) than by CE (Mean: 3.1+0.8%), especially in cases of the Hb(S) variant.

A good agreement was observed between HPLC and CE in measuring percentages of Hb variants in subjects with beta thalassaemia trait, beta thalassaemia major, sickle cell anaemia, Hb(D) Punjab, Hb(D) homozygous and sickle/β-thalassaemia. However, in cases diagnosed with Hb(D) Iran trait, HPLC demonstrated significantly higher Hb(A2) levels and failed to identify any Hb(D) Iran fraction. In contrast, CE showed a significantly lower Hb(A2) and a higher percentage of Hb(D) Iran variant as identified separately in a distinct zone. Mohanty *et al*.[15] also reported that in Hb(D) Iran cases, HPLC shows prominent Hb(A2) peaks, which should direct the clinician to suspect the presence of another Hb variant.

One outstanding feature observed of the CE method in the present study was the ability to obtain a clean measurement of Hb(A2) and Hb(E) in patients with Hb(E). In contrast, HPLC did not detect Hb(E). Similar results have been reported by Chopra *et al*.[16] who suggested that Hb(A2) cannot be accurately quantified in the presence of Hb(E) by HPLC BioRad. Hb(E) results from a mutation that introduces a splice site in exon 1, it produces fewer Hb(E) β chains and a picture of β-thalassemia.[17] However, routine screening methods, including alkaline and acid gel and currently approved HPLC methods, have not been able to sufficiently separate Hb(E) from Hb(A2) to allow for this measurement in routine chemistry laboratories.[18] Tandem mass spectrometry has been able to measure Hb(A2) in the presence of Hb(E).[19]

Table-III: Comparison between High Performance Liquid Chromatography (HPLC) and Capillary Electrophoresis (CE) in Detecting Hemoglobin Variants in Subjects with Different Haemoglobinopathies (n=90)

Hemoglobinopathy	N	Analyzer	Hb Variant %					
			Hb(A)	Hb(A2)	Hb(F)	Hb(S)	Hb(D)	Hb(E)
Beta Thalassaemia Trait	50	HPLC	93.24±1.07	5.77±1.08	0.99±1.6	--	--	--
		CE	93.29±1.02	5.69±1.01	1.02±0.12	--	--	--
		p-value	0.793	0.709	0.348	--	--	--
Beta Thalassaemia Major	12	HPLC	--	6.27±1.75	93.73±1.75	--	--	--
		CE	--	6.05±1.69	93.94±1.69	--	--	--
		p-value	--	0.76	0.77	--	--	--
Sickle Cell Anaemia	04	HPLC	--	1.8±0.68	9.15±4.62	89.05±5.27	--	--
		CE	--	1.9±0.88	9.77±5.59	88.35±6.23	--	--
		p-value	--	0.76	0.86	0.87	--	--
Sickle Cell Trait	03	HPLC	53.97±0.68	2.13±0.4	8.57±2.24	35.33±3.25	--	--
		CE	53.03±0.35	1.46±0.57	9.33±3.25	36.16±4.01	--	--
		p-value	0.10	0.17	0.75	0.79	--	--
Hb D Punjab Trait	12	HPLC	61.48±1.07	1.66±0.58	0.86±0.07	--	35.94±0.80	--
		CE	61.77±1.02	1.57±0.59	0.87±0.84	--	35.70±0.64	--
		p-value	0.50	0.71	0.69	--	0.43	--
Hb D homozygous	01	HPLC	--	--	3.6	--	96.4	--
		CE	--	--	3.8	--	96.2	--
		p-value	--	--	--*	--	--*	--
Sickle/β- Thalassaemia	02	HPLC	--	5.25±0.64	13±4.10	81.75±4.74	--	--
		CE	--	4.85±0.49	13.15±4.73	82±5.23	--	--
		p-value	--	0.56	0.98	0.96	--	--

** cannot be computed because SD = zero since only 01 case has been diagnosed*

Haemoglobinopathies are preventable, though not treatable, for which implementation of an appropriate diagnostic method with in-depth understanding is required.[20] Early haemoglobinopathies screening helps reduce affected births in endemic countries like Pakistan. Hence, efforts are made to enhance the efficiency of the screening process. The motive behind conducting this study was that a small number of such studies, so far, have been done in Pakistan. The results of this study add to the information in the existing pool of literature by providing statistical evidence about the efficiency of screening devices and help in effective decision-making and improved healthcare delivery.

CONCLUSION

This study concludes that the capillary electrophoresis method can identify and quantify haemoglobin species consistent with existing HPLC methods. Moreover, capillary electrophoresis is capable of identifying haemoglobin variants Hb(D) and Hb(E) in cases of the Hb(D) Iran trait and Hb(E) trait, which are not picked up by HPLC, making CE a more reliable method. As the utilization of CE grows for distinguishing haemoglobin variants, we suggest it be used as the first line of assessment in the majority of diagnostic laboratories.

Conflict of Interest: None.

Authors' Contribution

Following authors have made substantial contributions to the manuscript as under:

SA & AMA: Data acquisition, data analysis, drafting the manuscript, critical review, approval of the final version to be published.

JZ & RM: Study design, data interpretation, drafting the manuscript, critical review, approval of the final version to be published.

SZ & MK: Conception, data acquisition, drafting the manuscript, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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